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Commentary

RNAi technologies in agricultural biotechnology: The Toxicology Forum 40th Annual Summer Meeting

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ABSTRACT

During the 40th Annual Meeting of The Toxicology Forum, the current and potential future science, regulations, and politics of agricultural biotechnology were presented and discussed. The meeting session described herein focused on the technology of RNA interference (RNAi) in agriculture. The general process by which RNAi works, currently registered RNAi-based plant traits, example RNAi-based traits in development, potential use of double stranded RNA (dsRNA) as topically applied pesticide active ingredients, research related to the safety of RNAi, biological barriers to ingested dsRNA, recent regulatory RNAi science reviews, and regulatory considerations related to the use of RNAi in agriculture were discussed. Participants generally agreed that the current regulatory framework is robust and appropriate for evaluating the safety of RNAi employed in agricultural biotechnology and were also supportive of the use of RNAi to develop improved crop traits. However, as with any emerging technology, the potential range of future products, potential future regulatory frameworks, and public acceptance of the technology will continue to evolve. As such, continuing dialogue was encouraged to promote education of consumers and science-based regulations.

1. Introduction (Sherman)

The mission of The Toxicology Forum is to encourage open dialogue on human health and environmental issues that drive public concerns, academic involvement, industry action and regulatory decision making. The dialogue at their meetings facilitates conflict resolution, identifies research gaps, drives research agendas, and promotes sound regulatory and policy decision making (www.toxforum.org). This manuscript summarizes the second half of a day-long session on Agricultural Biotechnology at the 40th Annual Summer Meeting of The Toxicology Forum, which focused on RNA interference (RNAi) and its current and potential role in improving crop yields and nutritional quality. The first half of the day-long session focused on transgenic proteins in agricultural biotechnology and is summarized in the preceding companion manuscript (Sherman et al., 2015).

2. Introduction to the transformative technology of RNAi: how RNAi is being used in agricultural biotechnology (Munyikwa)

Many natural processes in eukaryotic organisms (plants, insects, animals, and nematodes etc.) such as the regulation of gene expression, suppression of invading viruses, and overall protection of the genome have been shown to be mediated by small RNAs. This occurs via a process now universally called RNAi (Fire et al., 1998;

Abbreviations: Ct, cycle threshold; dsRNA, double stranded RNA; EFSA, European Food Safety Authority; FDA, Food and Drug Administration; FIFRA, Federal Insecticide, Fungicide, and Rodenticide Act; GE, genetically engineered; GMO, genetically modified organism; miRNA, microRNA; MVs, microvesicles; NGO, non-governmental organization; PCR, polymerase chain reaction; PIP, plant-incorporated protectant; PPV, plum pox virus; RISC, RNA-induced silencing complex; RNAi, RNA interference; SAP, Scientific Advisory Panel; siRNA, small interfering RNA; ssRNA, single stranded RNA; USEPA, United States Environmental Protection Agency; USDA-ARS, United States Department of Agriculture-Agricultural Research Service.

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Brodersen and Voinnet, 2006; Ghildiyal et al., 2008; Jones-Rhoades et al., 2006; Mallory and Vaucheret, 2006; Huvenne and Smagghe, 2010). The term RNAi was popularized by Fire and Mello following their Nobel Prize winning work in Science or Medicine (2006) which demonstrated the potent effects of double stranded RNA (dsRNA) in *Caenorhabditis elegans* (Fire et al., 1998) and initiated intense research to understand the mechanisms underlying RNAi and its potential uses.

The basis of RNAi involves gene suppression at the transcription level or post transcriptional level. Suppression of gene expression is initiated by long dsRNAs that can emanate internally within or are introduced from outside a cell. A generalized description of the RNAi pathway is shown in Fig. 1. The long dsRNAs are cleaved by endonucleases such as Dicer and the Dicer-like proteins, to produce small interfering RNAs (siRNAs) which are double stranded RNAs that are on average 21–27 base pairs in length. The siRNAs complex with other proteins into an RNA Induced silencing complex (RISC) which unwinds and separates the two strands allowing for the specific base pairing between the small RNA and the targeted mRNA. This leads to reduction in the copy number of the specific mRNA by enzyme cleavage or lower protein concentration by suppression of translation. Ultimately the corresponding protein and its associated function are suppressed or eliminated within that organism's cells (Hamilton and Baulcombe, 1999; Huntzinger and Izaurralde, 2011). Other small RNAs that originate internally within cells e.g. microRNA (miRNA) and trans-acting RNA (tasiRNA) are also associated with the RNAi process in various organisms (Vazquez et al., 2004; Wilson and Doudna, 2013).

The mechanism of RNAi shows some differences amongst eukaryotes, including: 1) the requirements for nearly perfect complementarity for plant miRNAs as compared to animal miRNAs, and 2) the existence of an intercellular spreading process in plants, fungi and nematodes that has not yet been observed in insects or vertebrates (Tomari and Zamore, 2005; Miller et al., 2012). The intercellular spreading process involves amplification of the initial small pool of siRNAs by RNA-dependent RNA polymerase allowing for accumulation and intercellular spread of the RNAi molecules, via plasmodesmata and phloem in plants, or transmembrane type proteins such as SID1 in worms (Mittelbrunn and Sánchez-Madrid, 2012; Hunter et al., 2006; Zhang and Ruvkun, 2012).

The ability to suppress mRNA expression has created numerous new opportunities for the development of beneficial RNAi applications for the pharmaceutical and agricultural areas (Baum et al., 2007, Wu et al., 2014). For the latter, products that utilize the RNAi mode of action have the potential to offer new and complementary insect, bacteria, fungal, viral, and weed control solutions that have a degree of potency and selectivity beyond what has been possible to date using conventional pesticides. These products can be designed to precisely target specific mRNAs in a targeted species for which they are developed while leaving other organisms unaffected. The RNAi based products in development will be useful for the control of resistant populations of plant pests due to their unique mode of action. The agricultural industry is developing these products due to their high potential for having improved safety profiles and excellent specificity.

The delivery of products that utilize the RNAi mode of action as a biocontrol may occur as spray, drench, or granular applications. Alternatively the RNAi mode of action can be initiated as a plant-incorporated protectant (PIP) that is produced by a stably integrated transgene. A growing number of *in planta* RNAi-based events have been developed, reviewed, and received approval by international regulatory agencies, as shown in Table 1 below. These

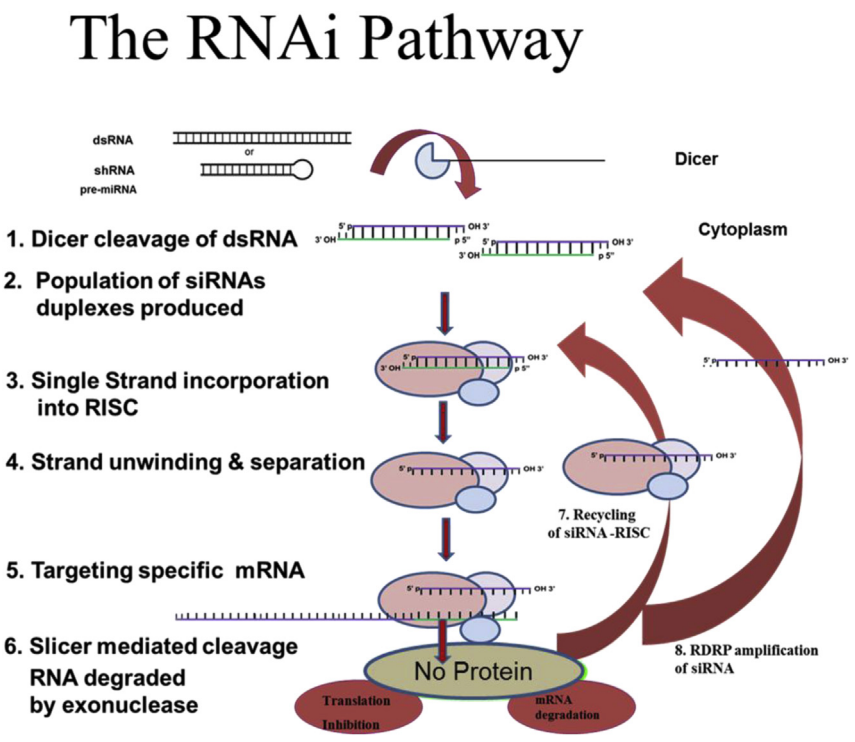

Fig. 1. The RNAi Pathway. Diagram shows a generalized view of the RNAi pathway: (**1**) long dsRNAs - derived from a) internally from microRNA (miRNAs), trans-acting RNA (tasiRNA)and micro RNA (miRNA) or b) externally from invading viruses, introduced dsRNA -are cleaved by the endonuclease called Dicer to small double stranded interfering RNAs (siRNAs) that are on average 21–27 nt in size (**2**). The siRNAs duplexes generated complex with other proteins into an RNA Induced Silencing Complex (RISC) (**3**) which unwinds and separates the two strands (**4**) The single strand siRNA-RISC complex targets a specific mRNA by specific Watson-Crick base pairing between the siRNA and the targeted mRNA (**5**) This leads to the elimination of the specific mRNA by enzyme cleavage or suppression of translation (**6**) Ultimately the corresponding proteins and associated function is eliminated within that organism's cells. Recycling of the siRNA-RISC complex occurs (**7**) as well in plants, fungi and nematodes (but not vertebrates) where there is a process of amplification of the small pool of siRNAs by RNA-Dependent RNA polymerase (**8**) that results in accumulation and intercellular spread of the RNAi molecules. (Note: Would Prefer Color).

Table 1
In planta RNAi products that have been reviewed and approved by at least one international regulatory agency.

Older products	New products/in development
•Tomatoes—Flavr Savr™ Tomato Delayed ripening—Monsanto (1994)	•Corn—High lysine—Renessen (2005)
•Squash—Virus resistance—Monsanto (1994)	•Plum—Resistance to plum pox virus (PPV)—USDA_ARS (2007)
	•Soybean—Modified Oil/Fatty acid—Dupont-Pioneer (2009)
	• Papaya -Resistance to viral infection, ring spot virus (PRSV)—University of Florida (2008)
• Papaya—Resistance to papaya ring spot virus (PRSV)—Cornell University and University of Hawaii (1996).	• Soybean - Modified Oil/Fatty acid—Monsanto (2011)
• Soybean—High oleic acid—DuPont Pioneer (1997)	• Amylopectin potato—BASF (2010)
• New Leaf Potato® Y—Monsanto (1998)	• Brazilian golden mosaic virus resistant beans—Embrapa—(2011)
• New Leaf Potato® Plus—Monsanto (1998)	• Reduced Lignin Alfalfa—Monsanto and Forage Genetics (2013)
	• Innate Potato—J.R. Simplot (2014)
	• Arctic™ "Golden Delicious" Apple—Okanagan Specialty Fruits (2015)

events encompass a wide variety of plant species from corn to potato as well as a range of traits including virus resistance and oil composition modification (e.g., lower trans-fats) in soybean oil (Frizzi and Huang, 2010).

Sprayable RNAi-based biocontrol products are still in development. The United States Environmental Protection Agency (US EPA) broadly anticipates that these products will fall under four categories, namely: 1) direct control agents; 2) resistance factor repressors; 3) developmental disruptors; and 4) growth enhancers (EPA, 2014). These RNA-based biocontrol products will likely be used outdoors on field crops and nurseries as well as indoors in storage facilities, green houses, etc.

The development of crop protectants that leverage the naturally occurring RNAi mode of action adds an additional agricultural productivity tool that shares the vast history of exposure to, and consumption of, small RNAs.

3. Are ingested small RNAs from plants significantly absorbed by humans? (Chan)

The past fifteen years of scientific discovery has offered a wealth of information about the biological activity of small non-coding RNA molecules with RNAi activity in eukaryotic organisms. In lower eukaryotes such as worms and insects (Issa et al., 2005; Newmark et al., 2003; Timmons and Fire, 1998), ingestion of exogenous RNAs allows for uptake and transfer of these molecules systemically (Feinberg and Hunter, 2003; Fire et al., 1998). However, ingestion of chemically synthesized RNA molecules designed for RNAi in larger mammals has proven to be an exceedingly challenging route of administration for effective uptake and biological activity, driven by the inherent instability of naked RNA oligonucleotides, the harsh environment of the gastrointestinal tract, and the lack of an identified specific molecular transporter in the gut for access to the systemic circulation. Recently, the discovery of the stability of extracellular microRNAs has re-invigorated a debate about their hypothesized ability to survive the milieu of the mammalian intestinal tract and transfer systemically to the host organism (Fig. 2). Notably, extracellular miRNAs have been discovered in a variety of body fluids. Also referred to as secreted or released miRNAs, these molecules are stable and can be packaged within microvesicles (MVs) or partnered with lipoproteins and RNA-binding molecules. They may be taken up by recipient cells where, if present in great enough quantity, can suppress target gene expression [as reviewed in (Creemers et al., 2012)].

In 2012, Zhang et al. reported that orally ingested, diet-derived miRNAs frequently are taken up by the mammalian gut and into systemic circulation (Zhang et al., 2012a). Their study focused on MIR168a, a miRNA in plants and rice which they reported can directly regulate the expression of the low-density lipoprotein receptor adapter protein 1 (LDLRAP1) in the liver and thus affect LDL cholesterol metabolism. Their team found high serum expression of MIR168a, as well as other miRNAs (e.g., MIR156 and MIR166A), in Chinese persons that maintained consistent ingestion of rice. Zhang and colleagues also reported that mice ingesting a diet of rice (carrying endogenous MIR156a, MIR166a, and MIR168a) displayed higher levels of these miRNAs in their serum and several solid organs (Zhang et al., 2012a). Finally, mice fed with a diet of fresh rice for a week displayed elevation of MIR168a in the liver and lower expression of LDLRAP1. A study by Wang and colleagues also detected a broad spectrum of exogenous nucleic acids in human blood using next-generation sequencing (Wang et al., 2012). However, in contrast to Zhang and colleagues, they did not examine dietary intake and MIR 168a was reported at an exceedingly low concentration.

Subsequent work has put the generalizability of these findings into question (Fig. 3). The Chan laboratory studied three conserved plant-derived miRNAs (MIR156A, MIR159A, and MIR169A) present in fruits as well as one conserved mammalian miRNA (miR-21) (Snow et al., 2013). These miRNAs were measured in human plasma, in plasma of mice that had been fed a diet with high levels of the three plant-derived miRNAs, and in plasma from mice genetically null for miR-21 that had been fed a diet with high levels of miR-21. All four exogenous miRNAs were detectable in some cases, but at concentrations of less than one copy per cell, making canonical miRNA activity unlikely. These concentrations differed from the high levels of MIR168a reported by Zhang and colleagues (approximately 850 copies per cell) (Zhang et al., 2012a).

The findings of the Chan laboratory were consistent with those of Witwer et al. (Witwer et al., 2013) who reported on the plasma miRNA content in pigtailed macaques following oral ingestion of a plant miRNA-enriched shake. In serial plasma samples taken post-ingestion, none of the plant-derived miRNAs chosen for study were detectable except for MIR160 – but only at low levels and not dependent upon shake ingestion. A third study published by Dickinson and colleagues attempted to replicate the study by Zhang et al. more directly (Dickinson et al., 2013). In that case, feeding of rice-containing diets at two different incorporation levels allowed for, at best, trace detection of plant-derived miRNAs in plasma that did not correlate with levels of dietary consumption. Furthermore, when examining LDLRAP1 and LDL cholesterol expression in these mice, the authors concluded that fasting and an unbalanced nutritional intake, not diet-derived miRNAs, drove the alterations in LDL originally reported by Zhang et al. (Zhang et al., 2012a).

Notably, the detectability of trace levels of exogenous miRNAs in mammalian plasma appears to be a reproducible finding across independent reports. However, discrepancies have emerged regarding the actual level of exogenous miRNA expression in recipient organisms that can realistically be achieved through delivery from a typical mammalian diet alone. Specifically, in order to deliver a biologically relevant amount of a given miRNA systemically throughout the human body as reported originally by Zhang

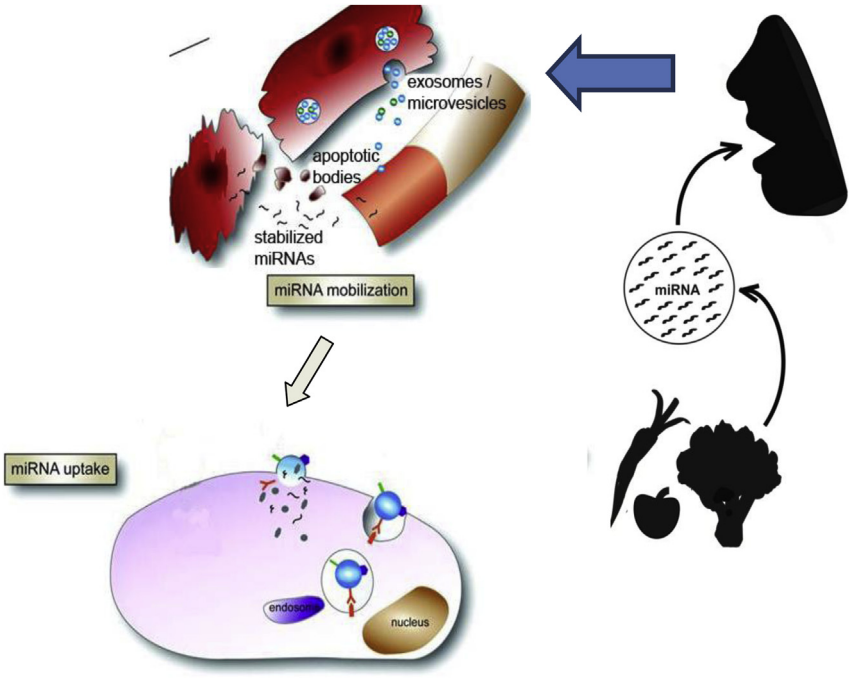

Fig. 2. Hypothesis of ingestion and uptake of diet-derived miRNAs in mammals. Given the stability of *ex vivo* miRNAs, it has been proposed that these molecules can survive the mammalian gastrointestinal milieu after ingestion. Given the growing literature describing the uptake of extracellular miRNAs into recipient cells, a hypothesis has emerged that these molecules can be transported through the gut into the systemic circulation and into recipient tissues in sufficient quantities to exert biological functions in canonical or non-canonical fashion. Images are adapted from Cottrill and Chan (2014), Gupta et al. (2010) with publisher permission.

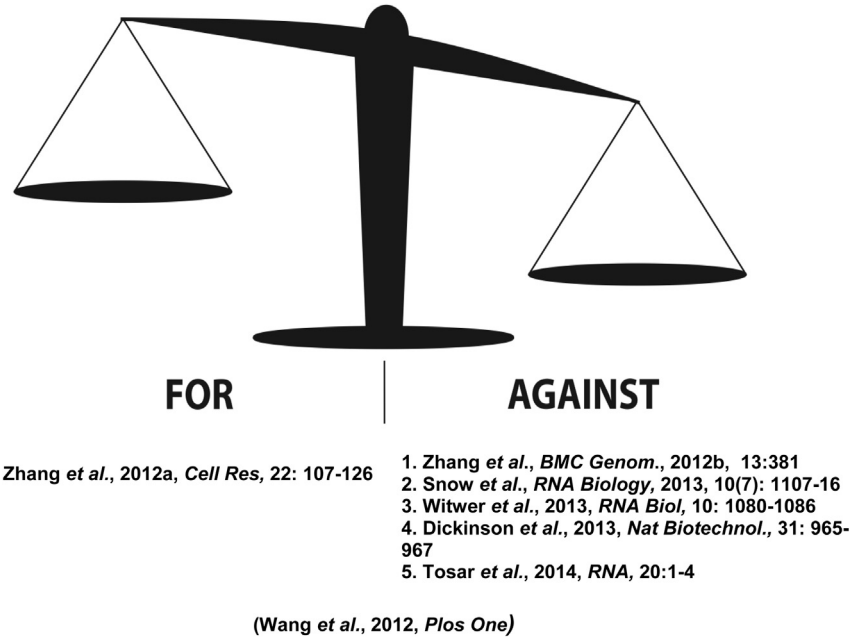

Fig. 3. Current evidence regarding transfer of diet-derived miRNAs to recipient mammals. For: Exogenous miRNAs have been reported to have gene regulatory effects in one case (Zhang et al., 2012a). Neutral: Exogenous RNAs have been detected in plasma and serum (Wang et al., 2012), Against: Experimental attempts to replicate oral uptake of miRNAs in mammals have been unsuccessful, even in a direct replication of the experimental conditions under which MIR168a was first reported to be taken up by rice-fed mice (Dickinson et al., 2013; Snow et al., 2013; Witwer et al., 2013). In these studies, reported levels of exogenous miRNAs in mammalian serum and plasma have been inconsistent and often at low concentrations not subject to dose-dependent ingestion. The extremely high sensitivity of quantitative PCR or next generation sequencing platforms can allow for detection of contaminating miRNAs in human samples (Tosar et al., 2014; Zhang et al., 2012b), thus facilitating false-positive readings of exogenous miRNAs. Reprinted with permission.

and colleagues (Zhang et al., 2012a), it has been calculated that one would need to consume the equivalent of approximately 1670 kg of cantaloupe (Snow et al., 2013). This assumes 10^6 copies of a specific plant-derived miRNA per mg of fruit, a minimum copy number of 100 copies per cell for canonical target gene repression, and 10^{13} cells in an average human body. Petrick and colleagues calculated a similarly unattainable dietary consumption rate when recently reporting on the safety of genetically-modified crops (Petrick et al., 2013). It remains conceivable that diets carrying concentrated amounts of miRNA may facilitate increased delivery. However, based on dosage levels alone, endogenous miRNAs present in normal human diets are not numerous enough to attain high enough levels in the bloodstream and recipient tissue to promote canonical gene regulatory functions.

Another key argument in this debate is the use of exceedingly sensitive technologies such as polymerase chain reaction (PCR) or next-generation sequencing to detect single copies of diet-derived miRNAs in recipient organisms. The occasional (and possibly non-specific (Witwer et al., 2013)) amplification of a plant sequence at high cycle threshold (Ct) values (Snow et al., 2013; Witwer et al., 2013) or fractional or single-digit high throughput sequencing reads per million of a single plant miRNA (Wang et al., 2012) have been noted in human plasma. However, an all too common problem of contaminating plant miRNAs has been reported by multiple groups in recent analyses of library preparation for next-generation sequencing (Zhang et al., 2012b) and particularly may have affected Zhang and colleagues' original work (Tosar et al., 2014). As such, future studies interrogating the delivery of diet-derived miRNAs should be conducted with extraordinary rigor to avoid such common pitfalls (Witwer and Hirschi, 2014).

So, where do we go from here? Theoretically, gastrointestinal disease, genetic conditions, or ingested substances could change gut permeability for exogenous miRNAs (and other molecules), and potentially more efficient uptake could increase the chances for biological activity in the recipient subject. Alternatively, one could envision that uptake may be improved by specialized packaging; and if certain tissue niches within the body can act as repositories for diet-derived miRNAs, these putative mechanisms could raise exogenous miRNA concentrations to more biologically relevant levels. Non-canonical actions of exogenous miRNAs (i.e., acting as signaling ligands) are also conceivable which may require a lower concentration for biological function. Additional studies are ongoing worldwide to interrogate these possibilities. However, recent dietary miRNA studies demonstrate definitively the absence of generalized uptake of dietary miRNAs for canonical antisense gene regulatory functions (Dickinson et al., 2013; Snow et al., 2013; Witwer et al., 2013). They further suggest that previous findings of uptake of exogenous miRNAs may have been affected by false-positives due to contaminating signals when using exceptionally sensitive techniques of miRNA measurements. Thus, while unanswered questions still remain in this field, convincing and reproducible evidence of uptake and delivery of diet-derived miRNAs at biologically relevant levels has yet to emerge.

4. Mammalian biological barriers to absorption of ingested dsRNA (Petrick)

In addition to the quantitative experimental evidence casting doubt on the activity and uptake of ingested small RNAs as discussed above, there are extensive biological barriers to systemic absorption and biodistribution of dsRNAs (Petrick et al., 2013). The weight of the evidence indicates the limited potential for physiologically-relevant absorption or biodistribution of diet-derived dsRNAs (Table 2). There is a history of safe consumption of dsRNA in our diets as evidenced by the presence of dsRNAs in all plant and animal derived foods that we eat. This includes plant dsRNAs with exact sequence matches to human genes/transcripts (Ivashuta et al., 2009; Frizzi et al., 2014; Jensen et al., 2013). Empirical evidence supporting the conclusion that diet-derived dsRNA are of little health concern is also provided by pharmaceutical research related to developing drugs based on RNAi technologies, where efforts to achieve oral efficacy of candidate dsRNA sequences in suppressing candidate target genes have largely been unsuccessful. This is not surprising considering there is generally very low ($\leq$1%) bioavailability of oligonucleotide therapeutics (reviewed in Petrick et al. (2013)). The conclusion of the general safety of diet-derived dsRNA has also recently been articulated by the Food Standards Australia New Zealand which stated: "There is no scientific basis for suggesting that small dsRNAs present in some GM foods have different properties or pose a greater risk than those already naturally abundant in conventional foods" (http://www.foodstandards.gov.au/consumer/gmfood/Pages/Response-to-Heinemann-et-al-on-the-regulation-of-GM-crops-and-foods-developed-using-gene-silencing.aspx last accessed June 24, 2015).

The history of safe consumption of dsRNA and the absence of efficacious oral RNA therapeutics, on the market or under development, is believed to largely result from multiple and redundant biological barriers to absorption and/or biodistribution. Gastrointestinal, cellular, and systemic distribution barriers are multifaceted (Table 2). Additionally, relatively rapid systemic clearance and degradation (e.g. within minutes) of circulating dsRNA (Thompson et al., 2012; Christensen et al., 2013) further limits the potential for ingested dsRNA to elicit an effect in the consuming organism. The appropriateness and applicability of the existing safety assessment framework for biotechnology-derived crops to those harnessing RNAi was emphasized based on the weight of the evidence for ingested RNA safety and the robustness of the existing case-by-case comparative safety assessment approach.

To further investigate the potential for dsRNA to be absorbed from the gut and distributed in biologically relevant concentrations, a proof of concept 28-day oral toxicity study was performed using dsRNAs targeting vacuolar ATPase, a sequence that is effective in controlling corn rootworms (Baum et al., 2007) but which was designed to have 100% sequence identity to the mouse ortholog (mice were the test species). The study design was adapted from OECD test guideline 407, but also included gene expression analysis as a measure for potential suppression of the target gene (since conference, data published in Petrick et al. (2015)). A 218 bp dsRNA or a pool of four 21-mer vATPase siRNAs elicited no adverse effects in either male or female mice after 28 days of exposure at the highest doses of 64 mg/kg and 48 mg/kg, respectively. These exposure levels are greater than 1,000,000-fold higher than anticipated human exposures from biotech crops, based on one such crop currently under development that utilizes RNA for insect control, and thus represents very large margins of exposure. In addition, no meaningful differences were noted in the level of vATPase mRNA expression in the brain, liver, kidney, stomach, duodenum, ileum, and spleen. As such, even at concentrations vastly greater than present in a normal diet, after daily oral ingestion for 28-days, dsRNAs with 100% homology to a mammalian transcript within the test species were unable to elicit adverse effects in mice or meaningfully impact the expression of the targeted gene.

The above results (since published as Petrick et al. (2015)) also shed light on the potential utility of bioinformatics as a tool for risk assessment of dsRNAs from RNAi-based crop traits; that is, sequence matches are not predictive of hazard. Together with a history of safe consumption of dsRNA in the diet and biological barriers, the high-dose vATPase dietary study indicates that sequence matches do not accurately predict a safety hazard in mammals - even when the dsRNA shares an exact match with endogenously expressed mRNA. As such, bioinformatics can identify putative matches, but does not appear to predict human health hazards and, thus, does not appear to be informative as a risk assessment tool.

5. Human health considerations for RNAi-Based pesticides: reflections on the USEPA SAP and EFSA panel deliberations (Witwer)

5.1. USEPA SAP

5.1.1. Background

Enforcement of the Federal Insecticide, Fungicide, and Rodenticide Act of 1947 (FIFRA) was transferred from the United States

Table 2
Safety of exogenous dsRNA in higher organisms.

Evidence Category	Supporting evidence
History of Safe Consumption	• Small RNAs and long dsRNAs with identity to human and animal transcripts are safely consumed in staple crops. • RNAi is not new to agriculture and underlies many domesticated crop phenotypes, as well as approved biotech crop traits
Efficacy of oral RNAi pharmaceuticals	• ≤1% oral bioavailability of oligo therapeutics • Direct injection, formulation, and stabilizing modifications needed for systemic activity • siRNA drugs are extensively metabolized and are cleared by the kidneys within minutes after i.v. dosing • Candidate RNA drugs have been safely administered at doses of up to 200 mg/kg i.v. in rats (Thompson et al., 2012)
Barriers to systemic distribution of ingested RNA	• Saliva • Stomach acid • Pancreatic nucleases • Intestinal epithelium and mucosa • Vascular endothelium • Blood/systemic nucleases • Rapid renal filtration (of any absorbed dsRNA)
Barriers to intracellular absorption and distribution	• Plasma membrane • Endosomal sequestration • Lysosomal degradation

Department of Agriculture to the US Environmental Protection Agency (USEPA) upon institution of the latter in 1970. In order to gain the advice of experts on various topics coming before the EPA, a FIFRA Scientific Advisory Panel (SAP) was later formed per the Federal Advisory Committee Act of 1972. The SAP includes seven permanent members who are nominated by federal entities such as the National Institutes of Health and the National Science Foundation. Holding approximately five to seven thematic meetings each year, the SAP is augmented by *ad hoc* members of the Science Review Board who are experts in areas relevant to the topic of each panel. By law, each meeting must be public and provide members of the public with the opportunity to present comments. Minutes must be released within 90 days of the event. It should be noted that the panelists are not required to form a consensus, and the advice of the FIFRA-SAP may guide but does not constitute or determine policy.

5.1.2. RNAi SAP

A FIFRA-SAP on "RNAi technology as a pesticide: Problem formulation for human health and ecological risk assessment" was convened at US EPA headquarters on January 28, 2014 and included three charge questions related to human health, as follows, and four pertaining to environmental considerations.

Assigned to various charge questions were both *ad hoc* and permanent SAP members. Of the nine members of the panel, seven were assigned exclusively to either human health (Table 3) or environment questions (not addressed here). As per guidelines, charge question responses were prepared by assigned panelists only and could not be shared with other panelists until the day of the meeting. In contrast with the usual FIFRA-SAP two-day format, this meeting was only one day in length, allowing less time for panelists to discuss viewpoints and finalize responses accordingly.

5.1.3. Human health considerations

Following a public comment session, primary respondents to the charge questions entered their answers. The respondents to the human health questions first took up the topic of potential off-target effects of RNAi effectors in mammals. Bioinformatics tools and substantial genomic coverage allow rapid assessment of potential binding sites of RNAi molecules in non-target organism transcriptomes. However, these tools also return many false positives, and despite the presence of many predicted binding sites of plant RNA in mammalian genomes, there is a history of safe exposure to plants and their expressed dsRNAs in the diet.

The question of off-target effects is in any case irrelevant if there is no functional exposure. Respondents emphasized the many barriers to uptake and function of dietary RNA, from RNases that degrade both single- and double-stranded RNA, to rapid clearance of bloodstream RNA by the kidneys, and the lack of confirmation of a high-profile report (Zhang et al., 2012a) that humans take up regulation-relevant levels of microRNAs. Numerous attempts to replicate these results, both published and unpublished, have found no significant uptake of dietary RNA (discussed above in Sections 2 and 3). These negative findings appeared to be consistent with the understanding that many species—and all mammals, as far as is known—do not have the molecular machinery to take up environmental RNA efficiently and incorporate it into RNA regulatory pathways, with or without amplification. Investigation of non-dietary routes of exposure, such as contact or inhalation exposure, might require further experimentation, as might dietary exposure of individuals with medical conditions predisposing to "leaky gut." However, independent calculations suggested that even 100% uptake and distribution of dietary RNA into functional cellular RNAi complexes could not result in per-cell copy numbers sufficient to regulate native messenger RNAs at physiologic levels of food intake (Snow et al., 2013; Petrick et al., 2013; and discussed above). The likelihood of "non-canonical" effects of ingested RNA, including innate immune system stimulation, or saturation of the native RNAi machinery, was also discussed. These effects, to the extent they might occur, would appear to require even higher levels of exposure.

Differences in the perception of abundance of plant-incorporated RNAi effector molecules were apparent amongst panelists. One member referred to "unprecedented" levels of RNA exposure and several were under the impression that the RNAi plant incorporated protectants must be highly overexpressed *in planta*. The question of abundance was thus posed to industry representatives, who stated that the RNA in question was typically found at very low abundance and was not among the most abundant RNAs in the plant. Interestingly, in then-unpublished experiments that were presented to the panel to establish the rapid environmental degradation of RNAi protectants, RNA was almost undetectable *in planta*, requiring the addition of exogenous RNA to achieve reliable readings in the persistence assays. The effectiveness of pesticidal RNAi mechanisms depends not on abundance of the RNAi precursor, but on the ability of the ingesting organism to take up dsRNA into cells and process it into short RNAi effectors.

5.1.4. Conclusions

Overall, with the aforementioned potential exceptions of specialized exposure routes or medical conditions, there was

Table 3
USEPA FIFRA SAP Human Health-Related Charge Questions and Assigned Panel Members. A = Ad hoc SAP panel member; P=Permanent SAP panel member.

Human health charge questions/Considerations	Assigned panel members
1) Please discuss the nature and extent of uncertainty in the specificity of long sequences of dsRNA targeted at pest species, if bioinformatic analysis shows no significant similarity to mammalian genes.	Brian Gregory (A), Brenda Oppert (A), Ken Witwer (A), Barry Delclos (P), James McManaman (P)
2) Based on data indicating degradation of the majority of dsRNA in the digestive system, please discuss the strengths and limitations in concluding there will not be significant adsorption of dsRNA with possible mammalian effects on oral exposure.	Brian Gregory (A), Brenda Oppert (A), Ken Witwer (A), Barry Delclos (P), James McManaman (P)
3) To what extent does the specific structure of dsRNA, if it is super coiled or in a hairpin structure, make it more likely to survive degradation in the gut and lead to possible mammalian effects with oral exposure?	Brian Gregory (A), Brenda Oppert (A), Ken Witwer (A)

consensus among the respondents that functional uptake of RNAi effectors by humans is unlikely and inconsistent with the available evidence (Table 2). This stood in contrast with the tenor of some of the environmental responses, which called into question the applicability of the current regulatory framework to evaluate potential environmental effects of plant-incorporated RNAi.

5.2. EFSA workshop

The European Food Safety Authority (EFSA) held a two-day workshop on the topic "Risk assessment considerations for RNAi-based GM plants" on June 4–5, 2014, in Brussels, Belgium. The first day of the meeting featured lectures on RNAi molecular biology, applications, and risk assessment by academic scientists such as Andrew Fire, co-discoverer of RNAi (Fire et al., 1998), and both government and industry scientists. The second day of the meeting was devoted to workshops on "Molecular characterization," "Food/feed risk assessment," and "Environmental risk assessment," which included participants from activist organizations as well.

5.2.1. Hypothetical unknowns versus known toxicities

During the day of lectures, Andrew Fire acknowledged past missteps by scientists in presuming the safety of the odd compound or technology that was ultimately found to have harmful side effects, but also gave the analogy of the homeowner standing on her roof, threatened by rising waters in a hurricane but refusing to climb into a rescue helicopter [because it is perceived as unsafe]. RNAi technology, one might conclude, could be an important tool in achieving food security, one that could be much safer because of its potential to be more specific and less toxic than conventional chemical pesticides.

5.2.2. Dietary RNA-mediated regulation in humans?

Presenter Gunter Meister had recently published a method for achieving knockdown in mammalian cells (Hannus et al., 2014). This protocol used multiple siRNAs to minimize concentrations of individual siRNA molecules and avoids off-target effects. The process essentially mimics the normal processing of dsRNA in non-mammalian organisms that are capable of importing dsRNA and processing it into a pool of siRNAs, and suggests that the extremely low levels of individual RNAi effectors (e.g., siRNAs) in ingested plants are unlikely to have off-target effects in mammals due to the impact of dilution with siRNA pools.

A questioner asked about survival and function of a plant miRNA or plant miRNA-protein complex in a mammalian cell. It was deemed unlikely that an uncomplexed small RNA would survive long enough in the extracellular or intracellular environment to be incorporated into a functional RISC in a recipient cell. Instead, regardless of being found or not in exosomes, plant miRNAs or siRNAs must be protected by plant proteins such as Argonaute-containing complexes. To achieve function in a mammalian cell, a protective complex would have to 1) escape digestion and dissociation in the gut, in circulation, and in the recipient cell; 2) be imported into the recipient cell cytoplasm intact; and 3) either function in coordination with evolutionarily distant machinery in the recipient cell, or transfer its RNA component to a mammalian RISC. It was mentioned that uncomplexed small RNAs are a molecular dead-end and have a short half-life, and that there is no evidence inter-RISC transfer could occur, even between two mammalian complexes.

What of the possibility that more stable dsRNA is taken up by ingesting mammals and subsequently processed? There is no evidence that foreign dsRNA is efficiently imported by mammalian cells or processed and amplified in a manner similar to what occurs in *C. elegans*. Furthermore, it was mentioned that plants do not process mammalian precursor RNAs. While it might be useful to perform additional experiments, there is no reason to assume that mammalian machinery would successfully process plant siRNA precursor molecules.

Ralph Scorza of USDA-ARS reported on an RNAi-based plum he developed, known as "Honeysweet," which is resistant to the plum pox virus (PPV). Interestingly, the same RNAs that confer resistance in this plant are found at much higher levels in the virus-infected "organic" fruits than in their transgenic counterparts. In the case of virus resistance, then, RNAi technology can actually *reduce* the exposure of the consumer to specific RNA molecules.

Chen-Yu Zhang, the corresponding author of controversial reports claiming dsRNA uptake by mammals (Zhang et al., 2012a), presented plant-based RNAs not as a threat, but rather as an opportunity for novel therapies of human disease. However, the chance of either harm or benefit was largely dismissed in another presentation that reviewed the stoichiometry of dietary RNA exposure, concluding like the FIFRA-SAP human health respondents that physiologic intake and uptake of dietary RNA is inconsistent with what is known about RNAi in mammalian cells.

5.3. Comparison: FIFRA-SAP and EFSA workshop

At the Toxicology Forum, it was mentioned that the USEPA SAP, while including sharply different and sometime contradictory viewpoints, seemed to place relative emphasis on scientific evidence, while the EFSA Workshop was set up to feature both scientific and more activism-driven positions. The conclusions of the EFSA Workshop were correspondingly equivocal, but did discard some postulated hazards of RNAi technology, such as the possibility that ingested RNA would saturate the mammalian RNA regulatory machinery (Table 4). Both the EPA and EFSA meetings raised the interest and mostly unexplored question of how the microbiome might interact with ingested RNA.

6. US FDA regulatory perspectives (Choudhuri)

The regulatory agencies and their respective regulatory

Table 4
Comparison of general consensus reached by USEPA SAP and EFSA panel.

Issue	EPA	EFSA
Biologically significant uptake from mammalian gut	No	Unknown
Functional Consequences and potential area for further study	Unlikely, but PiP-specific studies may be needed to increase level of certainty	Consensus on the potential for off-target effects not reached
	Absorption in compromised/diseased gut may be possible.	Saturation of endogenous RNAi machinery is unlikely
	Uncertainty regarding absorption after spray applications when compared to PIPs	Immune stimulation may be possible
	Uncertainty regarding the need for dermal or inhalation tests	Potential effects of inhaled dsRNA by workers or the public are uncertain
Effects on Microbiome	Unknown	Unknown

authorities, as well as the Food and Drug Administration (FDA) Centers involved in the safety evaluation of these GE (Genetically Engineered)[2] plant varieties developed using RNAi technologies and foods derived from them are discussed in the context of GE crop safety assessment within the companion manuscript on protein safety in GE crops (Sherman et al., 2015). The consultation process for GE plant varieties developed using RNAi technology is similar in scope to that employed for transgenic proteins. FDA's 1992 "Statement of Policy: Foods Derived from New Plant Varieties" (Federal Register Vol. 57 No. 104 Friday, May 29, 1992 p 22984; the 1992 policy statement) provides the framework for the safety evaluation. In other words, the 1992 policy statement provides the framework of safety evaluation of the GE plant varieties expressing proteins, as well as those in which the expression of a particular protein has been decreased ("knocked down") by RNAi mechanism.

One unique element to the safety assessment for RNAi is that no new protein is intended to be expressed from the RNAi sequence element of the inserted DNA, which is different from the earlier GE plant varieties in which the desired effect is typically gained from the expression of an introduced protein. Plants naturally express a plethora of small, noncoding, regulatory RNAs, which can be consumed by humans and animals if food crops expressing them are consumed.

The presentation concluded with examples of several GE crops developed using RNAi technology (e.g., reduced lignin (in forage) alfalfa; increased oleic acid soybean) for which FDA completed consultations. In the reduced lignin alfalfa, the expression of endogenous *CCOMT* gene is reduced via RNA interference resulting in a reduced level of CCOMT enzyme, which is involved in G lignin biosynthesis pathway. A reduced level of CCOMT enzyme results in decreased levels of G lignin and total lignin in forage. In the increased oleic acid soybean, the expression of *FATB1-A* and *FAD2-1A* genes is reduced via RNA interference. Because the products of these genes are involved in fatty acid biosynthesis pathway, the result of RNA interference is an altered fatty acid profile in soybean that includes marked increase in oleic acid level, and an associated decrease in linoleic acid level.

7. Discussion[3]

Meeting participants were supportive of the use of RNAi to develop improved crop traits, with several commenting that they were concerned that unless sufficient outreach efforts from industry, academics, and government are implemented, public perception of the technology could be inappropriately shaped by anti-biotech non-governmental organizations (NGOs) and hinder the acceptance of RNAi use in agricultural biotechnology.

Clarification was sought regarding how sequence matches to humans are being characterized in regulatory submittals, if structure–activity relationships are considered when developing products, and if building a library of potential mRNA targets would be helpful. In answering these questions, it was pointed out that there are public and private bioinformatics databases that are available and are being updated as new sequences are discovered. Bioinformatics is being used as a development tool to aid in product design, with an emphasis on limiting the potential for impacts to non-target organisms. However, the need for, and utility of, a bioinformatics assessment to inform the risk assessment of potential new RNAi plant traits in higher organisms (e.g., vertebrates) appears to be low due to the low concentrations in plants, the low mammalian bioavailability of ingested dsRNA, and the lack of correlation between sequence homology and safety in humans and other vertebrates. Review of the calculations presented by Dr. Chan provided a reasonable estimate of the low-dose threshold for ingested dsRNA to elicit a biological response in humans and the wide margin of safety calculated using the upper-bound expression levels of naturally occurring and transgenically expressed plant dsRNA.

Another question was raised regarding the use of bioinformatics to predict potential siRNA off-target effects via analyzing for matches between siRNAs and specific regions of target sequences (i.e. seed region matches), noting that some groups have demonstrated that by taking into consideration the thermodynamic properties of seed-target RNA duplexes, siRNAs can be designed to greatly minimize or eliminate potential off-target effects. The reply was that whereas the use of bioinformatics can be used to screen for off-target matches, the potency of siRNAs in eliciting in vivo effects is orders of magnitude lower than on target matches with mRNA. Furthermore, off-target seed region matches identified with bioinformatics are of questionable relevance to ingested RNAs, because gene expression changes induced by dsRNA with seed region homologies to mammalian genes are primarily observed in cases of *in vitro* transfection into cultured cells or direct injection of formulated/stabilized oligonucleotide drugs and are, therefore, not relevant to mammals ingesting plant expressed dsRNAs. This is because these model systems do not consider the multiple and redundant barriers to exogenous dsRNA exposures from the diet.

A participant asked how dsRNA for pesticide formulations would be commercially manufactured, specifically if it would be produced in bacteria. Currently, there are no commercial dsRNA spray products and their commercial introduction will require

[2] US FDA nomenclature for biotechnology-derived crops is Genetically Engineered (GE). Thus, through this section GE is used instead of GM.

[3] This paper is intended to describe the questions and views expressed at the meeting, but those views may not necessarily be those of all the authors and/or their employers.

innovation to reduce the cost of dsRNA manufacture. Different industrial scale dsRNA production/manufacturing processes are being investigated.

A question regarding how gut bacteria might be affected by dsRNA was raised. It is highly unlikely that the microbiome is impacted by dsRNAs, as humans have been consuming dsRNAs from plants and animals throughout history without any identified effects on the microbiome. Bacteria do not have an RNAi system, but do posses an analogous host defense system known as CRISR/Cas that is both phylogenetically and mechanistically different than eukaryotic RNAi and utilizes DNA, rather than RNA as an initiating signal (Horvath and Barrangou, 2010). Thus, dsRNAs have a very low probability to impact the microbiome due in part to mechanistic differences between RNAi and CRISPR/Cas found in prokaryotes.

The issue of inhalation exposures was brought up during discussions in relation to inhalation of plant particles containing dsRNA. In addition to there being a history of safe inhalation exposure of dsRNA contained in plant pollen and dust, in general the particle size of plant dust and pollen is larger than inhalable particles (generally considered to be < 10 μM). As such, inhaled pollen and plant dust particles are cleared from the upper airways and result in secondary oral exposures, not inhalation exposures.

In general, the relative stability of dsRNA, when compared to single-stranded RNA (ssRNA), was a novel concept for meeting participants. Certain RNases are specific for certain types of RNA and others act more broadly. While dsRNA tends to be more stable than ssRNA, it can be degraded by RNases, such as those found in blood and pancreatic secretions.

Technical questions related to the design of the oral vATPase study (since published as Petrick et al., 2015) were raised. One participant asked why there was not an i.v. positive control. Since the i.v. route of exposure is not an appropriate route of exposure for evaluating potential risks from ingesting dsRNA in plants, it is standard practice to only use oral dosing in food safety studies. The robust nature of the evaluated toxicology endpoints ensures that potential toxicity of the test substances was evaluated. Also, data developed by pharmaceutical companies indicates there is little potential for gene suppression from i.v. dosing of dsRNA, since it is rapidly cleared from the body after i.v. dosing. As such, there is no known positive control. Another participant questioned why the dose was so high (i.e., top doses of $\geq$48 mg/kg dsRNA for 28-days) in the vATPase studies. The doses were chosen to provide very large margins of exposure.

A question regarding the appropriateness of current chemical and/or biotech guidance for assessing potential hazards and risk from transgenically expressed dsRNA in plants was raised. It was felt that current toxicology testing guidelines and existing regulatory frameworks are robust, appropriate for evaluation of RNAi-based crop traits, and allow for a case-by-case approach to study design and product evaluation. However, if a new regulatory framework for RNAi is developed, it would be scientifically appropriate to determine the regulatory data needs by starting with problem formulation, rather than creating a study checklist as is currently done for conventional pesticides.

In summary, the session increased awareness regarding the current and potential future benefits of RNAi in agricultural biotechnology, the state-of-the-science regarding our understanding of RNAi, and the regulatory framework for evaluating the safety of GE plants. As with any emerging technology, the potential range of future products, potential future regulatory frameworks, and public acceptance of RNAi technology will continue to evolve. As such, continuing dialogue, such as promoted by The Toxicology Forum, was encouraged to promote educated consumers and science-based regulations.

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Transparency document

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References

Brodersen, P., Voinnet, O., 2006. The diversity of RNA silencing pathways in plants. Trends Genet. 22 (5), 268–280.
Baum, J.A., Bogaert, T., Clinton, W., Heck, G.R., Feldmann, P., Ilagen, O., Johnson, S., Plaetinck, G., Muniykwa, T., Pleau, M., Vaughn, T., Roberts, J., 2007. Control of Coleopteran insect pests through RNA interference. Nat. Biotechnol. 25 (11), 1322–1366.
Christensen, J., Litherland, K., Faller, T., van de Kerkhof, E., Natt, F., Hunziker, J., Krauser, J., Swart, P., 2013. Metabolism studies of unformulated internally [3H]-labeled short interfering RNAs in mice. Drug Metab. Dispos. 41, 1211–1219.
Cottrill, K.A., Chan, S.Y., 2014. Diet-derived MicroRNAs: separating the dream from reality. microRNA Diagn. Ther. 1, 46–57.
Creemers, E.E., Tijsen, A.J., Pinto, Y.M., 2012. Circulating microRNAs: novel biomarkers and extracellular communicators in cardiovascular disease? Circ. Res. 110, 483–495.
Dickinson, B., Zhang, Y., Petrick, J.S., Heck, G., Ivashuta, S., Marshall, W.S., 2013. Lack of detectable oral bioavailability of plant microRNAs after feeding in mice. Nat. Biotechnol. 31, 965–967.
EPA, 2014. RNAi Technology as a Pesticide: Problem Formulation for Human Health and Ecological Risk Assessment. FIFRA SAP, pp. 1–4.
Feinberg, E.H., Hunter, C.P., 2003. Transport of dsRNA into cells by the transmembrane protein SID-1. Science 301, 1545–1547.
Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., Mello, C.C., 1998. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 391, 806–811.
Frizzi, A., Huang, S., 2010. Tapping RNA silencing pathways for plant biotechnology. Plant Biotechnol. J. 8, 655–677.
Frizzi, A., Zhang, Y., Kao, J., Hagen, C., Huang, S., 2014. Small RNA profiles from virus-infected fresh market vegetables. J. Agric. Food Chem. 62, 12067–12074.
Ghildiyal, M., Seitz, H., Horwich, M.D., Li, C., Du, T., Lee, S., Xu, J., Kittler, E.L.W., Zapp, M.L., Weng, Zamore, P.D., 2008. Endogenous siRNAs derived from transposons and mRNAs in Drosophila somatic cells. Science 320 (5879), 1077–1081.
Gupta, S.K., Bang, C., Thum, T., 2010. Circulating microRNAs as biomarkers and potential paracrine mediators of cardiovascular disease. Circ. Cardiovasc Genet. 3, 484–488.
Hamilton, A.J., Baulcombe, D.C., 1999. A species of small antisense RNA in post-transcriptional gene silencing in plants. Science 286 (5441), 950–952, 29.
Hannus, M., Beitzinger, M., Engelmann, J.C., Weickert, M.T., Spang, R., Hannus, S., Meister, G., 2014. siPools: highly complex but accurately defined siRNA pools eliminate off-target effects. Nucleic Acids Res. 42 (12), 8049–8061.
Horvath, P., Barrangou, R., 2010. CRISPR/Cas, the immune system of bacteria and archaea. Science 327 (5962), 167–170.
Hunter, C.P., Winston, W.M., Molodowitch, C., Feinberg, E.H., Shih, J., Sutherlin, M., Wright, A.J., Fitzgerald, M.C., 2006. Systemic RNAi in Caenorhabditis elegans. Cold Spring Harb. Symp. Quant. Biol. 2006 (71), 95–100.
Huntzinger, E., Izaurralde, E., 2011. Gene silencing by microRNAs: contributions of translational repression and mRNA decay. Nat. Rev. Genet. 12 (2), 99–110.
Huvenne, H., Smagghe, G., 2010. Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: a review. J. Insect Physiol. 56 (3), 227–235.
Issa, Z., Grant, W.N., Stasiuk, S., Shoemaker, C.B., 2005. Development of methods for RNA interference in the sheep gastrointestinal parasite, Trichostrongylus colubriformis. Int. J. Parasitol. 35, 935–940.
Ivashuta, S.I., Petrick, J.S., Heisel, S.E., Zhang, Y., Guo, L., Reynolds, T.L., Rice, E.A., Roberts, J.K., 2009. Endogenous small RNAs in grain: semi-quantification and sequence homology to human and animal genes. Food Chem. Toxicol. 47, 353–360.
Jensen, P.D., Zhang, Y., Wiggins, B.E., Petrick, J.S., Zhu, J., Kerstetter, R.A., Heck, G.R., Ivashuta, S.I., 2013. Computational sequence analysis of predicted long dsRNA transcriptomes of major crops reveals sequence complementarity with human genes. GM Crops Food 4, 90–97.
Jones-Rhoades, M.W., Bartel, D.P., Bartel, B., 2006. MicroRNAS and their regulatory roles in plants. Annu. Rev. Plant Biol. 57, 19–53.
Mallory, A.C., Vaucheret, H., 2006. Functions of microRNAs and related small RNAs in plants. Nat. Genet. 38, S31–S36.
Miller, S.C., Miyata, K., Brown, S.J., Tomoyasu, Y., 2012. Dissecting systemic RNA interference in the red flour beetle Tribolium castaneum: parameters affecting the efficiency of RNAi. PLoS One 7, e47431.
Mittelbrunn, M., Sánchez-Madrid, F., 2012. Nat. Rev. Mol. Cell Biol. 13, 328–335.
Newmark, P.A., Reddien, P.W., Cebria, F., Sanchez Alvarado, A., 2003. Ingestion of bacterially expressed double-stranded RNA inhibits gene expression in

planarians. Proc. Natl. Acad. Sci. U. S. A. 100 (Suppl. 1), 11861–11865.
Petrick, J.S., Brower-Toland, B., Jackson, A.L., Kier, L.D., 2013. Safety assessment of food and feed from biotechnology-derived crops employing RNA-mediated gene regulation to achieve desired traits: a scientific review. Regul. Toxicol. Pharmacol. RTP 66, 167–176.
Petrick, J.S., Moore, W.M., Heydens, W.F., Koch, M.S., Sherman, J.H., Lemke, S.L., 2015. A 28-day oral toxicity evaluation of small interfering RNAs and a long double-stranded RNA targeting vacuolar ATPase in mice. Regul. Toxicol. Pharmacol. 71, 8–23.
Sherman, J.H., Choudhuri, S., Vicini, J.L., 2015. Transgenic proteins in agricultural biotechnology: the Toxicology Forum 40th Annual Meeting. Regul. Toxicol. Pharmacol. (in press).
Snow, J.W., Hale, A.E., Isaacs, S.K., Baggish, A.L., Chan, S.Y., 2013. Ineffective delivery of diet-derived microRNAs to recipient animal organisms. RNA Biol. 10, 1107–1116.
Thompson, J.D., Kornbrust, D.J., Foy, J.W.-D., Solano, E.C.R., Schneider, D.J., Feinstein, E., Molitoris, B.A., Erlich, S., 2012. Toxicological and pharmacokinetic properties of chemically modified siRNAs targeting p53 RNA following intravenous administration. Nucleic Acid. Ther. 22, 255–264.
Tomari, Y., Zamore, P.D., 2005. Perspective: machines for RNAi. Genes Dev. 19 (2005), 517–529.
Timmons, L., Fire, A., 1998. Specific interference by ingested dsRNA. Nature 395, 854.
Tosar, J.P., Rovira, C., Naya, H., Cayota, A., 2014. Mining of public sequencing databases supports a non-dietary origin for putative foreign miRNAs: underestimated effects of contamination in NGS. RNA 20, 754–757.
Vazquez, F., Vaucheret, H., Rajagopalan, R., Lepers, C., Gasciolli, V., Mallory, A.C., Hilbert, J.-L., Bartel, D.P., Créte, P., 2004. Endogenous trans-acting siRNAs regulate the accumulation of Arabidopsis mRNAs. Mol. Cell 16, 69–79.
Wang, K., Li, H., Yuan, Y., Etheridge, A., Zhou, Y., Huang, D., Wilmes, P., Galas, D., 2012. The complex exogenous RNA spectra in human plasma: an interface with human gut biota? PLoS One 7, e51009.
Wilson, R.C., Doudna, J.A., 2013. Molecular mechanisms of RNA interference. Annu. Rev. Biophys. 42, 217–239.
Witwer, K.W., Hirschi, K.D., 2014. Transfer and functional consequences of dietary microRNAs in vertebrates: concepts in search of corroboration: negative results challenge the hypothesis that dietary xenomiRs cross the gut and regulate genes in ingesting vertebrates, but important questions persist. BioEssays News Rev. Mol. Cell. Dev. Biol. 36, 394–406.
Witwer, K.W., McAlexander, M.A., Queen, S.E., Adams, R.J., 2013. Real-time quantitative PCR and droplet digital PCR for plant miRNAs in mammalian blood provide little evidence for general uptake of dietary miRNAs: limited evidence for general uptake of dietary plant xenomiRs. RNA Biol. 10, 1080–1086.
Wu, S.Y., Lopez-Berestein, G., Calin, G.A., Sood, A.K., 2014. RNAi therapies: drugging the undruggable. Sci. Transl. Med. 6 (240), 240ps7.
Zhang, L., Hou, D., Chen, X., Li, D., Zhu, L., Zhang, Y., Li, J., Bian, Z., Liang, X., Cai, X., Yin, Y., Wang, C., Zhang, T., Zhu, D., Zhang, D., Xu, J., Chen, Q., Ba, Y., Liu, J., Wang, Q., Chen, J., Wang, J., Wang, M., Zhang, Q., Zhang, J., Zen, K., Zhang, C.Y., 2012a. Exogenous plant MIR168a specifically targets mammalian LDLRAP1: evidence of cross-kingdom regulation by microRNA. Cell Res. 22, 107–126.
Zhang, Y., Wiggins, B.E., Lawrence, C., Petrick, J., Ivashuta, S., Heck, G., 2012b. Analysis of plant-derived miRNAs in animal small RNA datasets. BMC Genom. 13, 381.
Zhang, C., Ruvkun, G., 2012. New insights into siRNA amplification and RNAi. RNA Biol. 8, 1045–1049.